DESCRIPTION

METHOD AND REAGENT FOR THE INHIBITION OF TELOMERASE ENZYME

INS A1

Background Of The Invention

5   The present invention concerns compounds, compositions, and methods for the study, diagnosis, and treatment of conditions and diseases related to the level of telomerase enzyme.

The following is a brief description of the current understanding in the biology of telomerase and its components. The discussion is not meant to be complete and is
10   provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention.

The ribonucleoprotein enzyme telomerase consists of an RNA template subunit and one or more protein subunits including telomerase reverse transcriptase (TERT), which function together to direct the synthesis of telomeres. Telomeres exist as non-
15   nucleosome DNA/protein complexes at the physical ends of eukaryotic chromosomes. These capping structures maintain chromosome stability and replicative potential (Zakian, V. A., 1995, Science, 270, 1601-1607). Telomere structure is characterized by tandem repeats of conserved DNA sequences rich in G-C base pairs.   Additional conserved telomere elements include a terminal 3'-overhang in the G-rich strand and
20   non-histone structural proteins that are complexed with telomeric DNA in the nucleus. (Blackburn, "E., 1990, JBC., 265, 5919-5921.).   Observed shortening of telomeres coincides with the onset of cellular senescence in most somatic cell lines lacking significant levels of telomerase. This finding has had a profound impact on our views concerning the mechanisms of aging, age related disease, and cancer.

25   Conventional DNA polymerases are unable to fully replicate the ends of linear chromosomes (Watson, J. D., 1972, Nature, 239, 197-201). This inability stems from the 3' G-rich overhang that is a product of ribonuclease cleavage of the RNA primer used in DNA replication. The overhang prevents DNA polymerase replication since the recessed C-rich parent strand cannot be used as a template. Telomerase overcomes this
30   limitation by extending the 3' end of the chromosome using deoxyribonucleotides as substrates and a sequence within the telomerase RNA subunit as a template. (Lingner,

J., 1995, Science, 269, 1533-1534). As such, telomerase is considered a reverse transcriptase that is responsible for telomere maintenance.

Telomerase was first discovered by in *Tetrahymena thermophila* in 1985 (Greider, C. W., 1995, Cell, 43, 405-413). The RNA subunits and their respective genes were
5   later discovered and characterized in protozoa, budding yeast, and mammals. Genetic studies of these genes confirmed the role of telomerase RNA (TR) in determining telomere sequence by mutating genes which encode the telomeric RNA (Yu, G. L., 1990, Nature, 344, 126-132), (Singer, M. S., 1994, Science, 266, 404-409), (Blasco, M. A., 1995, Science, 269, 1267-1270). These studies showed that telomerase activity
10   parallels TR expression in protozoa, yeast and mice. However, the expression of human telomerase RNA (hTR) does not correlate well with telomerase activity in mammalian cells. Many human tissues express hTR but are devoid of telomerase activity (Feng, J., 1995, Science, 269, 1236-1241). Knockout mice, in which the mTR gene has been deleted from germline cells, have been shown to be viable for at least six generations.
15   Cells from later generations of these mice showed chromosomal abnormalities consistent with telomere degradation, indicating that mTR is necessary for telomere length maintenance, but is not required for embryonic development, oncogenic transformation, or tumor formation in mice (Blasco, M. A., 1997, Cell, 91, 25-34).

The first catalytically active subunit of telomerase (p123) was isolated from
20   *Euplotes aediculatus* along with another subunit (p43) and a 66-kD RNA subunit (Linger, J., 1996, Proc. Natl. Acad. Sci., 93, 10712-10717). Subsequent studies revealed telomerase catalytic subunit homologs from fission yeast (Est2p) and human genes (TRT1). The human homolog, TRT1 encoding hTERT, expressed mRNA with a strong correlation to telomerase activity in human cells (Nakamura, T. M., 1997,
25   Science, 277, 955-959). Reconstitution of telomerase activity with *in vitro* transcribed and translated hTERT and hTR, either co-synthesized or simply mixed, demonstrated that hTERT and hTR represent the minimal components of telomerase. Furthermore, transient expression of hTERT in normal diploid human cells restored telomerase activity, demonstrating that hTERT is the only component necessary to restore
30   telomerase activity in normal human cells (Weinrich, S. L., 1997, Nature Genetics, 17, 498-502). The introduction of telomerase into normal human cells using hTERT expression via transfection has resulted in the extension of life span in these cells. Such findings indicate that telomere loss in the absence of telomerase is the "mitotic clock"

that controls the replicative potential of a cell prior to senescence (Bodnar, A. G., 1998, Science, 279, 349-352).

Expression of telomerase is observed in germ cell and most cancer cell lines. These "immortal" cell lines continue to divide without shortening of their telomeres (Kim, N. W., 1994, Science, 266, 2011-2015). A model of tumor progression has evolved from these findings, suggesting a role for telomerase expression in malignant transformation. Successful malignant transformation in human cells was accomplished for the first time by ectopic expression of hTERT in combination with two oncogenes, SV40 large-T and H-ras. Injection of nude mice with cells expressing these oncogenes and hTERT resulted in rapid growth of tumors. These observations indicate that hTERT mediated telomere maintenance is essential for the formation of human tumor cells (Hahn, W. C., 1999, Nature, 400, 464-468).

Various methods have been developed to assay telomerase activity *in vitro*. The most widely used method to characterize telomerase activity is the telomeric repeat amplification protocol (TRAP). TRAP utilizes RT-PCR of cellular extracts to measure telomerase activity by making the amount of PCR target dependant upon the biochemical activity of the enzyme (Kim, N. W., 1997, Nucleic Acids Research, 25, 2595-2597).

A variety of animal models have been designed to assay telomerase activity *in vivo*. Inhibition of telomerase activity has been analyzed in rats via cell proliferation studies with MNU (N-methyl-N-nitosurea) induced mammary carcinomas in response to treatment with 4-(hydroxyphenyl)retinamide (4-HPR), a known inhibitor of mammary carcinogenesis in animal models and premenopausal women (Bednarek, A., 1999, Carcinogenesis, 20, 879-883). Additional studies have focused on the up-regulation of telomerase in transformed cell lines from animal and human model systems (Zhang, P. B., 1998, Leuk. Res., 22, 509-516), (Chadeneau, C., 1995, Oncogene, 11, 893-898), (Greenberg, R., 1999, Oncogene, 18, 1219-1226).

Human cell culture studies have been established to assay inhibition of telomerase activity in human carcinomas responding to various therapeutics. A human breast cancer model for studying telomerase inhibitors is described (Raymond, E., 1999, Br. J. Cancer, 80, 1332-1341). Human studies of telomerase expression as related to various other cancers are described including cervical cancer (Nakano, K., 1998, Am. J. Pathol,

153, 857-864), endometrial cancer (Kyo, S., 1999, Int. J. Cancer, 80, 60-63), meningeal carcinoma (Kleinschmidt-DeMasters, B. K., 1998, J. Neurol. Sci., 161, 124-134), lung carcinoma (Yashima, K., 1997, Cancer Reseach, 57, 2372-2377), testicular cancer in response to cisplatin (Burger, A. M., 1997, Eur. J. Cancer, 33, 638-644), and ovarian

5   carcinoma (Counter, C. M., 1994, Proc. Natl. Acad. Sci., 91, 2900-2904).

Particular degenerative and disease states that can be associated with telomerase expression modulation include but are not limited to:

- Cancer: Almost all human tumors have detectable telomerase activity (Shay, J. W., 1997, Eur. J. Cancer, 33, 787-791). Treatment with telomerase inhibitors

10   may provide effective cancer therapy with minimal side effects in normal somatic cells that lack telomerase activity. The therapeutic potential exists for the treatment of a wide variety of cancer types.

- Restinosis: Telomerase inhibition in vascular smooth muscle cells may inhibit restinosis by limiting proliferation of these cells.

15 - Infectious disease: Telomerase inhibition in infectious cell types that express telomerase activity may provide selective anti-infectious agent activity. Such treatment may prove especially effective in protozoan-based infection such as Giardia and Lesh Meniesis.

- Transplant rejection: Telomerase inhibition in endothelial cell types may

20   demonstrate selective immunnosuppressant activity. Activation of telomerase in transplant cells could benefit grafting success through increased proliferative potential.

- Autoimmune disease: Telomerase modulation in various immune cells may prove beneficial in treating diseases such as multiple sclerosis, lupus, and AIDS.

25 - Age related disease: Activation of telomerase expression in cells at or nearing senescence as a result of advanced age or premature aging could benefit conditions such as macular degeneration, skin ulceration, and rheumatoid arthritis.

The present body of knowledge in telomerase research indicates the need for methods to assay telomerase activity and for compounds that can regulate telomerase expression for research, diagnostic, trait alteration, animal health and therapeutic use.

5   Gaeta *et al.*, US patents No. 5,760,062; 5,767,278; 5,770,613 have described small molecule inhibitors of human telomerase RNA (hTR) subunit.

Blasco *et al.*, 1995, Science, 269, 1267-1270 describe the synthesis and testing of antisense oligonucleotides targeted against a specific region of the mouse telomerase RNA (mTR) subunit and reported reduction in telomerase activity in mice.

10   Bisoffi *et al.*, 1998, Eur. J. Cancer, 34, 1242-1249 have studied the down regulation of human telomerase activity by a retrovirus vector expressing antisense RNA targeted against the hTR RNA.

Norton *et al.*, 1996, Nature Biotechnology, 14, 615-619 have reported the use of a peptide nucleic acid (PNA) molecule targeting hTR RNA to down regulate telomerase activity in human immortal breast epithelial cells.

15   Yokoyama *et al.*, 1998, Cancer Research, 58, 5406-5410 have reported the synthesis and testing of hammerhead ribozyme constructs targeting hTR RNA resulting in a decrease in the telomerase activity in Ishikawa cells.

Henderson, European Patent Application No. 666,313-A2 describes methods of identifying and cloning hTR gene for use in gene therapy approaches for creating

20   aberrant telomeric sequences in transfected human tumor cells. A ribozyme based gene therapy approach to inhibit the expression of hTR gene is described as well. The intended result of such therapies involves incurred genetic instability based on non-native telomeric sequences resulting in rapid cell death of the treated cells.

West *et al.*, US patent No. 5,489,508 describe methods for determining telomere

25   length and telomerase activity in cells. Inhibitors of hTR RNA, including oligonucleotides and/or small molecules are described.

These foregoing approaches of targeting the telomerase RNA subunit (TR) may not be very beneficial, because as demonstrated by Feng *et al.*, (Feng, J., 1995, Science, 269, 1236-1241), telomerase activity in humans does not correlate well to hTR

30   concentration

Collins *et al.*, International PCT publication No. WO 98/01542 describes assays for the detection of telomerase activity. Four human telomerase subunit proteins are described called p140, p105, p48 and p43. In addition, hybridization probes and primers are described as inhibitors of telomerase gene function. Antibody based

5   inhibitors of telomerase protein subunits are described.

A more attractive approach to telomerase regulation would involve the regulation of human telomerase by modulating the expression of the protein subunits of the enzyme, preferably the reverse transcriptase (hTERT) subunit. Based of reconstitution experiments, hTERT and hTR represent the minimal components of telomerase. Since

10   hTR expression does not correlate well with telomerase activity in human cells and since many human cells express hTR without telomerase activity, targeting hTERT may prove more beneficial than targeting hTR. hTERT is the only component necessary to restore telomerase activity in normal human cells. A study in which the three major subunits of telomerase (hTR, TP1, and hTERT were assayed in normal and malignant

15   endometrial tissues determined that hTERT is a rate limiting determinant of enzymatic activity of human telomerase (Kyo, S., 1999, Int. J. Cancer, 80, 60-63). Additional protein subunits that have been isolated most likely serve only a structural role in telomerase activity, but may be important in enhancing the activity of the telomerase enzyme. As such, hTERT is one of the better targets for the ectopic regulation of

20   telomerase activity.

Cech *et al.*, International PCT publication No. WO 98/14593 describe compositions and methods related to hTERT for diagnosis, prognosis and treatment of human diseases, for altering proliferative capacity in cells and organisms, and for screening compounds and treatments with potential use as human therapeutics.

25   Cech *et al.*, International PCT publication No. WO 98/14592 describe nucleic acid and amino acid sequences encoding various telomerase protein subunits and motifs of *Euplotes aediculatus*, and related sequences from *Schizosaccharomyces*, *Saccharomyces* sequences, and human telomerase. The polypeptides comprising telomeric subunits and functional polypeptides and ribonucleoproteins that contain these

30   subunits are described as well. Cech *et al.*, International PCT Publication No. *WO 98/14592*, mentions in general terms the possibility of using antisense and ribozymes to down regulate the expression of human telomerase reverse transcriptase enzyme.

Summary Of The Invention

The invention features novel nucleic acid-based techniques [e.g., enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, 2-5A antisense chimeras, triplex DNA, antisense nucleic acids containing RNA cleaving chemical groups (Cook *et al.*, U.S. Patent 5,359,051)] and methods for their use to down regulate or inhibit the expression of telomerase enzyme.

In a preferred embodiment, the invention features use of one or more of the nucleic acid-based techniques to inhibit the expression of the genes encoding the protein subunits of the telomerase enzyme, preferably the catalytic subunit of the telomerase enzyme. Specifically, the invention features the use of nucleic acid-based techniques to specifically inhibit the expression of telomerase reverse transcriptase (TERT) gene.

In another preferred embodiment, the invention features the use of an enzymatic nucleic acid molecule, preferably in the hammerhead, NCH, G-cleaver and/or DNAzyme motif, to inhibit the expression TERT gene.

In another preferred embodiment, the invention features the inhibition or down regulation of telomerase activity by inhibiting or down regulating the expression of one or more activators of telomerase enzyme, such as protein encoded by *ras* gene. Such activator gene expression may be regulated by the use of nucleic acid-based techniques, such as enzymatic nucleic acid molecules and antisense oligonucleotides.

By "inhibit" it is meant that the activity of telomerase enzyme or level of RNAs or equivalent RNAs encoding one or more protein subunits of the telomerase enzyme is reduced below that observed in the absence of the nucleic acid. In one embodiment, inhibition with enzymatic nucleic acid molecule preferably is below that level observed in the presence of an enzymatically inactive or attenuated molecule that is able to bind to the same site on the target RNA, but is unable to cleave that RNA. In another embodiment, inhibition with antisense oligonucleotides is preferably below that level observed in the presence of for example, an oligonucleotide with scrambled sequence or with mismatches. In another embodiment, inhibition of TERT genes with the nucleic acid molecule of the instant invention is greater than in the presence of the nucleic acid molecule than in its absence. According to the invention, the activity of telomerase

enzyme or the level of RNA encoding one or more protein subunits of the telomerase enzyme is inhibited if it is at least 10% less, 20% less, 50% less, 75% less or even not active or present at all, in the presence of a nucleic acid of the invention relative to the level in the absence of such a nucleic acid.

5   As used herein, the term "telomerase activity" refers to enzyme activity that replicates, for example, the TTAGGG repeats at the ends of linear chromosomes. Telomerase activity is comprised by a ribonucleoprotein enzyme comprising one or more protein subunits and an RNA subunit. The enzymatic activity extends the 5'-recessed end of a linear chromosome using deoxyribonucleotides and an RNA sequence within the RNA subunit as a primer. Telomerase activity may be assayed as follows.

10

Samples to be assayed for telomerase activity are prepared by extraction into CHAPS lysis buffer (10mM Tris pH 7.5, 1mM $MgCl_2$, 1mM EGTA, 0.1 mM PMSF, 5mM -mercaptoethanol, 1mM DTT, 0.5% 3-[(3-cholamidopropyl)-dimethyl-amino]-1-propanesulfonate (CHAPS), 10% glycerol and 40 U/ml RNAse inhibitor (Promega, Madison, WI, U.S.A.). Cells are suspended in CHAPS lysis buffer and incubated on ice for 30 minutes, which allows lysis of 90-100% of cells. Lysate is then transferred to polyallomer centrifuge tubes and spun at 100,000 x g for 1 hour at 4 degrees C. The supernatant is the protein extract, and concentration ranges of 4-10 $\mu g/\mu l$ are suitable for telomerase assay. Extracts may be concentrated if necessary using a Microcon Microfilter 30 (Amicron, Beverly, MA U.S.A.) according to the manufacturer's instructions. Extracts may be stored frozen at –80 degrees C until assayed.

15

20

Telomerase may be assayed according to Kim and Wu, *Nucl. Acids Res.* 25: 2595-2597, incorporated herein by reference. Briefly, for the telomerase assay, 2µg of protein extract is used. The extract is assayed in 50µl of reaction mixture containing 0.1 µg TS substrate primer (5'-AATCCGTCGAGCAGAGTT-3', end-labeled using alpha-^{32}P-ATP and T4 polynucleotide kinase), 0.1µg ACX return primer(5'-GCGCGG[CTTACC]₃ CTAACC-3'), 0.1 µg NT internal control primer (5'-ATCGCTTCTCGGCCTTTT-3'), 0.01 micromol TSNT internal control template (5'-AATCCGTCGAGCAGAGTTAAAAGGCCGAGAACGAT-3'), 50 µM each deoxynucleoside triphosphate, 2 U of Taq DNA polymerase, and 2 µl CHAPS protein extract, all in 1X TRAP buffer (20 mM Tris (pH 8.3), 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.05% Tween 20). Each reaction is placed in a thermocycler block preheated to 30 C and incubated at 30 C for 10 minutes, then cycled for 27 cycles of 94 degrees C for 30 seconds, 60 degrees C for 30 seconds. Reaction products are separated

25

30

on a denaturing 8% polyacrylamide gel, followed by drying of the gel and autoradiography. The internal control (to control for possible Taq polymerase inhibition) generates a band of 36 nt. Comparison of radioactive signal integrated (*e.g.*, by phorphorimager analysis) for telomerase-extended bands with the radioactive signal

5 from a reaction performed with a known amount of quantification standard template (termed R8; 5'-AATCCGTCGAGCAGAGTTAG [GGTTAG]$_7$-3') allows expression of telomerase activity as an absolute value. The absolute value = TPG (total product generated) =[(TP-TPí)/TI]/[(R8-B)/RI)] x 100, where TP = telomerase products from test extract, TPí = telomerase products from a heat-inactivated (75 C, 10 minutes)

10 extract reaction, TI = the signal from the internal control, R8 = the signal from the R8 qualification standard template reaction, B = signal from a lysis buffer-only blank reaction, and RI = the internal control value for the reaction containing R8 template and NT and TSNT control primers. TPG values of 0-10,000 are possible, with the linear range being from approximately 1 to 1000 TPG. The range of 1 to 1000 TPG

15 encompasses the minimum and maximum levels of telomerase activity in most tumor samples tested, while non-tumor cells most often have no telomerase activity (TPG approximately zero).

An alternative telomerase assay, which does not employ PCR amplification, is described by Raymond et al. 1999, *Br. J. Cancer* 80: 1332-1341.

20 By "enzymatic nucleic acid molecule" it is meant an RNA molecule which has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave target RNA. That is, the enzymatic RNA molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. This complementary regions allow sufficient hybridization of

25 the enzymatic RNA molecule to the target RNA and thus permit cleavage. One hundred percent complementarity between RNA and the target gene or target RNA is preferred, but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA,

30 enzymatic RNA, catalytic DNA, aptazyme or aptamer-binding ribozyme, regulatable ribozyme, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity. The specific enzymatic nucleic acid molecules described in the instant application are not

meant to be limiting and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it have a specific substrate binding site which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate

5 binding site which impart a nucleic acid cleaving activity to the molecule (Cech *et al.,* U.S. Patent No. 4,987,071; Cech *et al.,* 1988, JAMA).

By "enzymatic portion" or "catalytic domain" is meant that portion/region of the enzymatic nucleic acid molecule essential for cleavage of a nucleic acid substrate (for example see Figure 1).

10 By "substrate binding arm" or "substrate binding domain" is meant that portion/region of a ribozyme which is complementary to (*i.e.,* able to base-pair with) a portion of its substrate. Generally, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 may be base-paired. Such arms are shown generally in Figure 1. That is, these arms contain sequences within a ribozyme

15 which are intended to bring ribozyme and target RNA together through complementary base-pairing interactions. The ribozyme of the invention may have binding arms that are contiguous or non-contiguous and may be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides and of sufficient length to stably interact with the target RNA; specifically 12-100 nucleotides; more specifically

20 14-24 nucleotides long. If two binding arms are chosen, the design is such that the length of the binding arms are symmetrical (*i.e.,* each of the binding arms is of the same length; *e.g.,* five and five nucleotides, six and six nucleotides or seven and seven nucleotides long) or asymmetrical (*i.e.,* the binding arms are of different length; *e.g.,* six and three nucleotides; three and six nucleotides long; four and five nucleotides long;

25 four and six nucleotides long; four and seven nucleotides long; and the like).

By DNAzyme is meant, an enzymatic nucleic acid molecule lacking a 2'-OH group. In particular embodiments the enzymatic nucleic acid molecule may have an attached linker(s) or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups.

30 By "sufficient length" is meant an oligonucleotide of greater than or equal to 3 nucleotides, 5 nucleotides, 7 nucleotides, 9 nucleotides or even 12 nucleotides.

By "stably interact" is meant, interaction of the oligonucleotides with target nucleic acid (e.g., by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions).

By "equivalent" RNA to telomerase enzyme is meant to include those naturally occurring RNA molecules having homology (partial or complete) to nucleic acid sequences encoding telomerase proteins or encoding for proteins with similar function as telomerase in various organisms, including human, rodent, primate, rabbit, pig, protozoans, fungi, plants, and other microorganisms and parasites. The equivalent RNA sequence also includes in addition to the coding region, regions such as 5'-untranslated region, 3'-untranslated region, introns, intron-exon junction and the like.

By "homology" is meant the nucleotide sequence of two or more nucleic acid molecules is partially or completely identical.

By "antisense nucleic acid" it is meant a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm et al., 1993 Nature 365, 566) interactions and alters the activity of the target RNA (for a review see Stein and Cheng, 1993 Science 261, 1004). Typically, antisense molecules will be complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule may bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule may bind such that the antisense molecule forms a loop. Thus, the antisense molecule may be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule may be complementary to a target sequence or both.

By "2-5A antisense chimera" it is meant, an antisense oligonucleotide containing a 5' phosphorylated 2'-5'-linked adenylate residues. These chimeras bind to target RNA in a sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease which, in turn, cleaves the target RNA (Torrence et al., 1993 Proc. Natl. Acad. Sci. USA 90, 1300).

By "triplex DNA" it is meant an oligonucleotide that can bind to a double-stranded DNA in a sequence-specific manner to form a triple-strand helix. Formation of such triple helix structure has been shown to inhibit transcription of the targeted gene (Duval-Valentin et al., 1992 Proc. Natl. Acad. Sci. USA 89, 504).

By "gene" it is meant a nucleic acid that encodes an RNA.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another RNA sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its target or complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., ribozyme cleavage, antisense or triple helix inhibition. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, CSH Symp. Quant. Biol. LII pp.123-133; Frier et al., 1986, Proc. Nat. Acad. Sci. USA 83:9373-9377; Turner et al., 1987, J. Am. Chem. Soc. 109:3783-3785. A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

At least seven basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Table I summarizes some of the characteristics of these ribozymes. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor of gene expression, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme.

The enzymatic nucleic acid molecule that cleave the specified sites in telomerase-specific RNAs represent a novel therapeutic approach to treat a variety of pathologic indications, including, cancer, tumorigenesis, restenosis and others.

In one of the preferred embodiments of the inventions described herein, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis delta virus, group I intron, group II intron or RNase P RNA (in association with an RNA guide sequence), *Neurospora* VS RNA, DNAzymes, NCH cleaving motifs, or G-cleavers. Examples of such hammerhead motifs are described by Dreyfus, *supra*, Rossi *et al.*, 1992, *AIDS Research and Human Retroviruses* 8, 183; of hairpin motifs by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, and Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; Chowrira & McSwiggen, US. Patent No. 5,631,359; of the hepatitis delta virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16; of the RNase P motif by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO. J.* 14, 363); Group II introns are described by Griffin *et al.*, 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; Pyle *et al.*, International PCT Publication No. *WO 96/22689*; of the Group I intron by Cech *et al.*, U.S. Patent 4,987,071 and of DNAzymes by Usman *et al.*, International PCT Publication No. WO 95/11304; Chartrand *et al.*, 1995, *NAR* 23, 4092; Breaker *et al.*, 1995, *Chem. Bio.* 2, 655; Santoro *et al.*, 1997, *PNAS* 94, 4262. NCH cleaving motifs are described in Ludwig & Sproat, International PCT Publication No. WO 98/58058; and G-cleavers are described in Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120 and Eckstein *et al.*, International PCT Publication No. WO 99/16871. Additional motifs such as the Aptazyme (Breaker *et al.*, WO 98/43993), Amberzyme (Class I motif, Figure 3; Beigelman *et al.*, U.S. Serial No. 09/301,511) and Zinzyme (Beigelman *et al.*, U.S. Serial No. 09/301,511) can also be used in the present invention. These specific motifs are not limiting in the invention and those skilled in the art will

recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the

5   molecule (Cech *et al.*, U.S. Patent No. 4,987,071).

In preferred embodiments of the present invention, a nucleic acid molecule, *e.g.*, an antisense molecule, a triplex DNA, or a ribozyme, is 13 to 100 nucleotides in length, *e.g.*, in specific embodiments 35, 36, 37, or 38 nucleotides in length (*e.g.*, for particular ribozymes or antisense). In particular embodiments, the nucleic acid molecule is 15-

10   100, 17-100, 20-100, 21-100, 23-100, 25-100, 27-100, 30-100, 32-100, 35-100, 40-100, 50-100, 60-100, 70-100, or 80-100 nucleotides in length. Instead of 100 nucleotides being the upper limit on the length ranges specified above, the upper limit of the length range can be, for example, 30, 40, 50, 60, 70, or 80 nucleotides. Thus, for any of the length ranges, the length range for particular embodiments has lower limit as specified,

15   with an upper limit as specified which is greater than the lower limit. For example, in a particular embodiment, the length range can be 35-50 nucleotides in length. All such ranges are expressly included. Also in particular embodiments, a nucleic acid molecule can have a length which is any of the lengths specified above, for example, 21 nucleotides in length.

20   In a preferred embodiment the invention provides a method for producing a class of nucleic acid –based gene inhibiting agents which exhibit a high degree of specificity for the RNA of a desired target. For example, the enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of target RNAs encoding telomerase proteins (specifically TERT gene) such that specific treatment of a disease or

25   condition can be provided with either one or several nucleic acid molecules of the invention. Such nucleic acid molecules can be delivered exogenously to specific tissue or cellular targets as required. Alternatively, the nucleic acid molecules (*e.g.*, ribozymes and antisense) can be expressed from DNA and/or RNA vectors that are delivered to specific cells.

30   By "highly conserved sequence region" is meant a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

The nucleic acid-based inhibitors of telomerase expression are useful for the prevention of the diseases and conditions including cancer, macular degeneration, restenosis, certain infectious diseases, transplant rejection and autoimmune disease such as multiple sclerosis, lupus, and AIDS; Age related disease such as macular degeneration, skin ulceration, and rheumatoid arthritis. and any other diseases or conditions that are related to the levels of telomerase in a cell or tissue.

By "related" is meant that the reduction of telomerase expression (specifically TERT gene) RNA levels and thus reduction in the level of the respective protein will relieve, to some extent, the symptoms of the disease or condition.

The nucleic acid-based inhibitors of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues ex vivo, or in vivo through injection, infusion pump or stent, with or without their incorporation in biopolymers. In preferred embodiments, the enzymatic nucleic acid inhibitors comprise sequences which are complementary to the substrate sequences in **Tables III-VII**. Examples of such enzymatic nucleic acid molecules also are shown in **Tables III to VII**. Examples of such enzymatic nucleic acid molecules consist essentially of sequences defined in these Tables.

In yet another embodiment, the invention features antisense nucleic acid molecules and 2-5A chimera including sequences complementary to the substrate sequences shown in **tables III to VII**. Such nucleic acid molecules can include sequences as shown for the binding arms of the enzymatic nucleic acid molecules in **Tables III to VII**. Similarly, triplex molecules can be provided targeted to the corresponding DNA target regions, and containing the DNA equivalent of a target sequence or a sequence complementary to the specified target (substrate) sequence. Typically, antisense molecules will be complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule may bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule may bind such that the antisense molecule forms a loop. Thus, the antisense molecule may be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule may be complementary to a target sequence or both.

By "consists essentially of" is meant that the active ribozyme contains an enzymatic center or core equivalent to those in the examples, and binding arms able to bind mRNA such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such cleavage. Thus, a core region may, for example, include one or more loop or stem-loop structures which do not prevent enzymatic activity. "X" in the sequences in Tables III and IV can be such a loop. A core sequence for a hammerhead ribozyme can be CUGAUGAG X CGAA where X=GCCGUUAGGC or other stem II region known in the art.

In another aspect of the invention, ribozymes or antisense molecules that cleave target RNA molecules and inhibit telomerase enzyme (specifically TERT) activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme or antisense expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes or antisense are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes or antisense. Such vectors might be repeatedly administered as necessary. Once expressed, the ribozymes or antisense bind to the target RNA and inhibit its function or expression. Delivery of ribozyme or antisense expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

By "patient" is meant an organism which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which the nucleic acid molecules of the invention can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed above. For example, to treat a disease or condition associated with the levels of telomerase enzyme, the patient may be treated, or other appropriate cells

may be treated, as is evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the described molecules, such as antisense or ribozymes can be used in combination with other known treatments to treat conditions or diseases

5   discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents to treat cancer.

In another preferred embodiment, the invention features nucleic acid-based inhibitors (*e.g.*, enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, 2-5A antisense chimeras, triplex DNA, antisense nucleic acids containing RNA cleaving

10   chemical groups) and methods for their use to down regulate or inhibit the expression of genes (*e.g.*, TERT) capable of progression and/or maintenance of cancer.

In another preferred embodiment, the invention features nucleic acid-based techniques (*e.g.*, enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, 2-5A antisense chimeras, triplex DNA, antisense nucleic acids containing RNA cleaving

15   chemical groups) and methods for their use to down regulate or inhibit the expression of TERT gene expression.

By "comprising" is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be

20   present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action

25   specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Other features and advantages of the invention will be apparent from the

30   following description of the preferred embodiments thereof, and from the claims.

Description Of The Preferred Embodiments

First the drawings will be described briefly.

Drawings

Figure 1 shows the secondary structure model for seven different classes of enzymatic nucleic acid molecules. Arrow indicates the site of cleavage. --------- indicate
5   the target sequence. Lines interspersed with dots are meant to indicate tertiary interactions. - is meant to indicate base-paired interaction. **Group I Intron**: P1-P9.0 represent various stem-loop structures (Cech *et al.*, 1994, *Nature Struc. Bio.*, 1, 273). **RNase P (M1RNA)**: EGS represents external guide sequence (Forster *et al.*, 1990, *Science*, 249, 783; Pace *et al.*, 1990, *J. Biol. Chem.*, 265, 3587). **Group II Intron:** 5'SS
10   means 5' splice site; 3'SS means 3'-splice site; IBS means intron binding site; EBS means exon binding site (Pyle *et al.*, 1994, *Biochemistry*, 33, 2716). **VS RNA:** I-VI are meant to indicate six stem-loop structures; shaded regions are meant to indicate tertiary interaction (Collins, International PCT Publication No. WO 96/19577). **HDV Ribozyme: :** I-IV are meant to indicate four stem-loop structures (Been *et al.*, US Patent
15   No. 5,625,047). **Hammerhead Ribozyme: :** I-III are meant to indicate three stem-loop structures; stems I-III can be of any length and may be symmetrical or asymmetrical (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527). **Hairpin Ribozyme:** Helix 1, 4 and 5 can be of any length; Helix 2 is between 3 and 8 base-pairs long; Y is a pyrimidine; Helix 2 (H2) is provided with a least 4 base pairs (*i.e.,* n is 1, 2, 3 or 4) and helix 5 can
20   be optionally provided of length 2 or more bases (preferably 3 - 20 bases, *i.e.,* m is from 1 - 20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (*i.e.,* r is $\geq$ 1 base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs (*e.g.,* 4 - 20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and
25   each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (*i.e.,* o and p is each independently from 0 to any number, *e.g.,* 20) as long as some base-pairing is maintained. Essential bases are shown as specific bases in the structure, but those in the
30   art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed from two separate molecules, *i.e.,* without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" $\geq$ is 2 bases. The connecting loop

can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "_____" refers to a covalent bond. (Burke *et al.*, 1996, *Nucleic Acids & Mol. Biol.*, 10, 129; Chowrira *et al.*, US Patent No. 5,631,359).

5 Figure 2 shows examples of chemically stabilized ribozyme motifs. **HH Rz,** represents hammerhead ribozyme motif (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527); **NCH Rz** represents the NCH ribozyme motif (Ludwig & Sproat, International PCT Publication No. WO 98/58058); **G-Cleaver,** represents G-cleaver ribozyme motif (Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120). **N** or **n,** represent independently a nucleotide which may be same or different and have complementarity to each other; **rI,** represents ribo-Inosine nucleotide; arrow indicates the site of cleavage within the target. Position 4 of the HH Rz and the NCH Rz is shown as having 2'-*C*-allyl moddification, but those skilled in the art will recognize that this position can be modified with other modifications well known in the art, so long as such modifications do not significantly inhibit the activity of the ribozyme.

15 Figure 3 shows an example of the Amberzyme ribozyme motif that is chemically stabilized (see for example Beigelman *et al.*, WO 99/55857; also referred to as Class I Motif).

Figure 4 shows an example of the Zinzyme A ribozyme motif that is chemically stabilized (see for example Beigelman *et al.*, WO 99/55857; also referred to as Class A Motif).

Mechanism of action of Nucleic Acid Molecules of the Invention

Antisense: Antisense molecules may be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in inhibition of peptide synthesis (Wu-Pong, Nov 1994, *BioPharm*, 20-33). The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules may also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm (Mukhopadhyay & Roth, 1996, *Crit. Rev. in Oncogenesis* 7, 151-190).

In addition, binding of single stranded DNA to RNA may result in nuclease degradation of the heteroduplex (Wu-Pong, *supra*; Crooke, *supra*). To date, the only backbone modified DNA chemistry which will act as substrates for RNase H are phosphorothioates and phosphorodithioates. Recently it has been reported that 2'-
5   arabino and 2'-fluoro arabino- containing oligos can also activate RNase H activity.

A number of antisense molecules have been described that utilize novel configurations of chemically modified nucleotides, secondary structure, and/or RNase H substrate domains (Woolf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, USSN 60/082,404 which was filed on April 20, 1998; Hartmann *et al.*,
10   USSN 60/101,174 which was filed on September 21, 1998) all of these are incorporated by reference herein in their entirety.

Triplex Forming Oligonucleotides (TFO):   Single stranded DNA may be designed to bind to genomic DNA in a sequence specific manner. TFOs are comprised of pyrimidine-rich oligonucleotides which bind DNA helices through Hoogsteen Base-
15   pairing (Wu-Pong, *supra*). The resulting triple helix composed of the DNA sense, DNA antisense, and TFO disrupts RNA synthesis by RNA polymerase. The TFO mechanism may result in gene expression or cell death since binding may be irreversible (Mukhopadhyay & Roth, *supra*)

2-5A Antisense Chimera: The 2-5A system is an interferon mediated mechanism
20   for RNA degradation found in higher vertebrates (Mitra *et al.*, 1996, *Proc Nat Acad Sci USA* 93, 6780-6785).   Two types of enzymes, 2-5A synthetase and RNase L, are required for RNA cleavage. The 2-5A synthetases require double stranded RNA to form 2'-5' oligoadenylates (2-5A).   2-5A then acts as an allosteric effector for utilizing RNase L which has the ability to cleave single stranded RNA. The ability to form 2-5A
25   structures with double stranded RNA makes this system particularly useful for inhibition of viral replication.

(2'-5') oligoadenylate structures may be covalently linked to antisense molecules to form chimeric oligonucleotides capable of RNA cleavage (Torrence, *supra*). These molecules putatively bind and activate a 2-5A dependent RNase, the
30   oligonucleotide/enzyme complex then binds to a target RNA molecule which can then be cleaved by the RNase enzyme.

Enzymatic Nucleic Acid: Seven basic varieties of naturally-occurring enzymatic RNAs are presently known. In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London,* B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing cleavage and ligation of phosphodiester linkages (Joyce, 1989, *Gene,* 82, 83-87; Beaudry *et al.,* 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.,* 1994, *TIBTECH* 12, 268; Bartel *et al.,*1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.,* 1995, *FASEB J,* 9, 1183; Breaker, 1996, *Curr. Op. Biotech.,* 7, 442; Santoro *et al.,* 1997, *Proc. Natl. Acad. Sci.,* 94, 4262; Tang *et al.,* 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, supra; Ishizaka et al., 1995, *supra*; Vaish *et al.,* 1997, *Biochemistry* 36, 6495; all of these are incorporated by reference herein). Each can catalyze a series of reactions including the hydrolysis of phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions.

Nucleic acid molecules of this invention will block to some extent telomerase protein expression (specifically TERT) and can be used to treat disease or diagnose disease associated with the levels of telomerase enzyme.

The enzymatic nature of a ribozyme has significant advantages, such as the concentration of ribozyme necessary to affect a therapeutic treatment is lower. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can be chosen to completely eliminate catalytic activity of a ribozyme.

Nucleic acid molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. Such enzymatic nucleic acid molecules can be targeted to virtually any RNA transcript, and achieved efficient cleavage *in vitro* (Zaug *et al.,* 324, *Nature* 429 1986 ; Uhlenbeck, 1987 *Nature* 328, 596; Kim et al., 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987; Dreyfus, 1988, *Einstein Quart. J. Bio. Med.,* 6, 92; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030, 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989; Santoro *et al.,* 1997 *supra*).

Because of their sequence specificity, *trans*-cleaving ribozymes show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* **30**, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* **38**, 2023-2037). Ribozymes can be designed to cleave specific RNA targets within the background of

5   cellular RNA. Such a cleavage event renders the RNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited.

Target sites

Targets for useful ribozymes and antisense nucleic acids can be determined as

10   disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468, and hereby incorporated by reference herein in totality. Other examples include the following PCT applications which concern inactivation of expression of disease-related genes: WO 95/23225, WO 95/13380, WO 94/02595, incorporated by

15   reference herein. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Ribozymes and antisense to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. The sequence of human TERT RNAs were screened for optimal enzymatic nucleic acid and antisense

20   target sites using a computer folding algorithm. Antisense, hammerhead, DNAzyme, NCH, or G-Cleaver ribozyme binding/cleavage sites were identified. These sites are shown in **Tables III to VII** (all sequences are 5' to 3' in the tables; X can be any base-paired sequence, the actual sequence is not relevant here). The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of enzymatic

25   nucleic acid molecule. While human sequences can be screened and enzymatic nucleic acid molecule and/or antisense thereafter designed, as discussed in Stinchcomb *et al.*, WO 95/23225, mouse targeted ribozymes may be useful to test efficacy of action of the enzymatic nucleic acid molecule and/or antisense prior to testing in humans.

Antisense, hammerhead, DNAzyme, NCH, or G-Cleaver ribozyme

30   binding/cleavage sites were identified. The nucleic acid molecules were individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the sequences fold into the appropriate secondary structure. Those nucleic acid molecules with unfavorable intramolecular interactions such as between the

binding arms and the catalytic core were eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity.

Antisense, hammerhead, DNAzyme, NCH, or G-Cleaver ribozyme binding/cleavage sites were identified and were designed to anneal to various sites in the

5 RNA target. The binding arms are complementary to the target site sequences described above. The nucleic acid molecules were chemically synthesized. The method of synthesis used follows the procedure for normal DNA/RNA synthesis as described below and in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-

10 2684; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than

15 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, antisense oligonucleotides, hammerhead or the hairpin ribozymes) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of RNA structure. Exemplary molecules of the instant invention were

20 chemically synthesized, and others can similarly be synthesized. Oligodeoxyribonucleotides were synthesized using standard protocols as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19, and is incorporated herein by reference.

The method of synthesis used for normal RNA including certain enzymatic

25 nucleic acid molecules follows the procedure as described in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting

30 example, small scale syntheses were conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.75 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. Table II outlines the amounts and the contact times of the reagents used in the synthesis

cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 15-fold excess (31 μL of 0.1 M = 3.1 μmol) of phosphoramidite and a 38.7-fold excess of S-ethyl tetrazole (31 μL of 0.25 M = 7.75

5 μmol) relative to polymer-bound 5'-hydroxyl was used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, were 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer; detritylation solution was 3% TCA in methylene chloride (ABI); capping was performed with 16%

10 N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile was used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from American International Chemical, Inc.

15 Deprotection of the RNA was performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide was transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL

20 of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, were dried to a white powder. The base deprotected oligoribonucleotide was resuspended in anhydrous TEA/HF/NMP solution (300 μL of a solution of 1.5 mL N-methylpyrrolidinone, 750 μL TEA and 1 mL TEA•3HF to provide a 1.4 M HF

25 concentration) and heated to 65 °C. After 1.5 h, the oligomer was quenched with 1.5 M NH_4HCO_3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide was transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO:1/1 (0.8 mL) at 65 °C for 15 min. The

30 vial was brought to r.t. TEA•3HF (0.1 mL) was added and the vial was heated at 65 °C for 15 min. The sample was cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution was loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA was detritylated with 0.5% TFA for 13 min. The cartridge was then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide was then eluted with 30% acetonitrile.

Inactive hammerhead ribozymes or binding attenuated control (BAC) oligonucleotides) were synthesized by substituting a U for G_5 and a U for A_{14} (numbering from Hertel, K. J., *et al.*, 1992, *Nucleic Acids Res.*, 20, 3252). Similarly, one or more nucleotide substitutions can be introduced in other enzymatic nucleic acid molecules to inactivate the molecule and such molecules can serve as a negative control.

The average stepwise coupling yields were >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96 well format, all that is important is the ratio of chemicals used in the reaction.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997 *Bioconjugate Chem.* 8, 204).

The nucleic acid molecules of the present invention are modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163). Ribozymes are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, Supra, the totality of which is hereby incorporated herein by reference) and are re-suspended in water.

The sequences of the ribozymes that are chemically synthesized, useful in this study, are shown in **Tables III to VII**. Those in the art will recognize that these sequences are representative only of many more such sequences where the enzymatic

portion of the ribozyme (all but the binding arms) is altered to affect activity. The ribozyme sequences listed in **Tables III to V and VII** may be formed of ribonucleotides or other nucleotides or non-nucleotides. Such ribozymes with enzymatic activity are equivalent to the ribozymes described specifically in the Tables.

5   Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases may increase their potency (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken et al., 1991 *Science* 253,

10   314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra*; all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules herein). Modifications which

15   enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired. (All these publications are hereby incorporated by reference herein).

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant

20   enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996

25   *Biochemistry* 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No. WO 92/07065; Perrault *et al. Nature* 1990, *344*, 565-568; Pieken *et al. Science* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *US Patent*

30   No. 5,334,711 and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702; Beigelman *et al.*,

International PCT publication No. WO 97/26270; Beigelman *et al.*, US Patent No. 5,716,824; Usman *et al.*, US patent No. 5,627,053; Woolf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.*, 1998 *Tetrahedron Lett.* 39, 1131; ; all of the references

5   are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without inhibiting catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid molecules of

10   the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, too many of these modifications may cause some toxicity. Therefore when designing nucleic acid molecules the amount of these internucleotide linkages should be

15   minimized. The reduction in the concentration of these linkages should lower toxicity resulting in increased efficacy and higher specificity of these molecules.

Nucleic acid molecules having chemical modifications which maintain or enhance activity are provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may

20   not be significantly lowered. Therapeutic nucleic acid molecules delivered exogenously must optimally be stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, nucleic acid molecules must be resistant to nucleases in order to function as effective

25   intracellular therapeutic agents. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19) incorporated by reference herein) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

Use of these the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple antisense or enzymatic nucleic acid molecules targeted to different genes, nucleic acid molecules coupled with known small molecule inhibitors, 5 or intermittent treatment with combinations of molecules (including different motifs) and/or other chemical or biological molecules)). The treatment of patients with nucleic acid molecules may also include combinations of different types of nucleic acid molecules.

Therapeutic nucleic acid molecules (e.g., enzymatic nucleic acid molecules and 10 antisense nucleic acid molecules) delivered exogenously must optimally be stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, these nucleic acid molecules must be resistant to nucleases in order to function as effective intracellular therapeutic agents. 15 Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

By "enhanced enzymatic activity" is meant to include activity measured in cells 20 and/or in vivo where the activity is a reflection of both catalytic activity and ribozyme stability. In this invention, the product of these properties is increased or not significantly (less that 10 fold) decreased in vivo compared to an all RNA ribozyme.

In yet another preferred embodiment, nucleic acid catalysts having chemical modifications which maintain or enhance enzymatic activity is provided. Such nucleic 25 acid is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or in vivo the activity may not be significantly lowered. As exemplified herein such ribozymes are useful in a cell and/or in vivo even if activity over all is reduced 10 fold (Burgin et al., 1996, Biochemistry, 35, 14090). Such ribozymes herein are said to "maintain" the enzymatic activity on all RNA ribozyme.

30 In another aspect the nucleic acid molecules comprise a 5' and/or a 3'- cap structure.

By "cap structure" is meant chemical modifications, which have been incorporated at the terminus of the oligonucleotide (see for example Wincott *et al.*, WO 97/26270, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or

5   localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminus (3'-cap) or may be present on both terminus. In non-limiting examples: the 5'-cap is selected from the group comprising inverted abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide, carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides;

10   modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate;

15   3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details see Beigelman *et al.*, International PCT publication No. WO 97/26270, incorporated by reference herein). In yet another preferred embodiment the 3'-cap is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic

20   nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-

25   inverted nucleotide moeity; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein). By the term

30   "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or

35   thymine.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group may be substituted or unsubstituted. When substituted the

5   substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino, or SH. The term also includes alkenyl groups which are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably it is a lower alkenyl of from 1 to 7 carbons, more

10   preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The term "alkyl" also includes alkynyl groups which have an unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups.

15   Preferably, the alkynyl group has 1 to 12 carbons. More preferably it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino or SH.

20   Such alkyl groups may also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group which has at least one ring having a conjugated p electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to

25   an alkyl group (as described above) covalently joined to an aryl group (as described above. Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen,

30   sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An

"amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located
5   at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International
10   PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*) all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into
15   nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, and others (Burgin *et al.*, 1996,
20   *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

25   By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position.

By "ribonucleotide" is meant a nucleotide with one of the bases adenine, cytosine, guanine, or uracil joined to the 1' carbon of   -D-ribo-furanose.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine,
30   uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

5   In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant $2'$-NH_2 or $2'$-O-NH_2, which may be modified or unmodified. Such modified groups are described, for example, in Eckstein et al., U.S. Patent 5,672,695 and Matulic-Adamic et al., WO 98/28317, respectively, which are both incorporated by reference in their entireties.

10   Various modifications to nucleic acid (*e.g.*, antisense and ribozyme) structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

15   Use of these molecules will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes (including different ribozyme motifs) and/or other chemical or biological molecules). The treatment of patients with nucleic acid molecules may also include combinations of different types of nucleic acid 20   molecules. Therapies may be devised which include a mixture of ribozymes (including different ribozyme motifs), antisense and/or 2-5A chimera molecules to one or more targets to alleviate symptoms of a disease.

Administration of Nucleic Acid Molecules

Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 25   1992, *Trends Cell Bio.*, 2, 139; and *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995 which are both incorporated herein by reference. Sullivan *et al.*, PCT WO 94/02595, further describes the general methods for delivery of enzymatic RNA molecules. These protocols may be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules may be administered to cells by a variety of methods 30   known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels,

cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, nucleic acid molecules may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, supra and Draper *et al.*, PCT WO93/23569 which have been incorporated by reference herein.

The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a patient.

The negatively charged polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the like.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or patient, preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation to reach a target cell (*i.e.*, a cell to which the

negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

5   By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively

10   charged polymers, *e.g.*, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation

15   which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as the cancer cells.

The invention also features the use of the composition comprising surface-

20   modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer an method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the

25   encapsulated drug (Lasic *et al. Chem. Rev.* 1995, **95**, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, **43**, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, **267**, 1275-1276; Oku *et al.,*1995, *Biochim. Biophys. Acta*, **1238**, 86-90). The long-circulating liposomes enhance the

30   pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to

conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 1995, **42**, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392; all of these are incorporated by reference herein). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen. All of these references are incorporated by reference herein.

The present invention also includes compositions prepared for storage or administration which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors which those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the present invention may also be administered to a patient in combination with other therapeutic compounds to increase the overall

therapeutic effect. The use of multiple compounds to treat an indication may increase the beneficial effects while reducing the presence of side effects.

Alternatively, certain of the nucleic acid molecules of the instant invention can be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985
5   *Science* 229, 345; McGarry and Lindquist, 1986 *Proc. Natl. Acad. Sci.* USA 83, 399; Scanlon *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992 *Antisense Res. Dev.*, 2, 3-15; Dropulic *et al.*, 1992 *J. Virol*, 66, 1432-41; Weerasinghe *et al.*, 1991 *J. Virol*, **65**, 5531-4; Ojwang *et al.*, 1992 *Proc. Natl. Acad. Sci. USA* 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*,
10   1990 *Science* 247, 1222-1225; Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Good *et al.*, 1997, *Gene* Therapy, 4, 45; all of the references are hereby incorporated in their totality by reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary
15   transcript by a ribozyme (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al*, 1992 *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993 *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994 *J. Biol. Chem.* 269, 25856; all of the references are hereby incorporated in their totality by reference herein).

20   In another aspect of the invention, RNA molecules of the present invention are preferably expressed from transcription units (see for example Couture *et al.*, 1996, *TIG.*, 12, 510) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus,
25   or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of nucleic acid molecules. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecule binds to the target mRNA. Delivery of nucleic
30   acid molecule expressing vectors could be systemic, such as by intravenous or intra-

muscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.*, 12, 510).

5   In one aspect the invention features, an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention is disclosed. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operable linked in a manner which allows expression of that nucleic acid molecule.

10   In another aspect the invention features, the expression vector comprises: a transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); c) a gene encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said gene is operably linked to said initiation region and said termination 15   region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the gene encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

          Transcription of the nucleic acid molecule sequences are driven from a promoter 20   for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that 25   the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993 *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990 *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that nucleic acid molecules, such as ribozymes expressed from such

promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993 *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992 *EMBO J.* 11, 4411-8; Lisziewicz *et al.*, 1993

5   *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8000-4; Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as ribozymes in cells (Thompson *et al.*, *supra*; Couture

10   and Stinchcomb, *1996, supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, US Patent No. 5,624,803; Good *et al.*, 1997, *Gene Ther.* 4, 45; Beigelman *et al.*, International PCT Publication No. *WO 96/18736*; all of these publications are incorporated by reference herein. The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian

15   cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra*).

In yet another aspect the invention features an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the

20   invention, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic

25   acid molecule. In another preferred embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an open reading frame; d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said open reading frame and said termination

30   region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In yet another embodiment the expression vector comprises: a) a transcription initiation region; b) a transcription termination region; c) an intron; d) a

gene encoding at least one said nucleic acid molecule; and wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation

5   region; b) a transcription termination region; c) an intron; d) an open reading frame; e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid

10   molecule.

Examples.

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

The following examples demonstrate the selection and design of Antisense,

15   hammerhead, DNAzyme, NCH, or G-Cleaver ribozyme molecules and binding/cleavage sites within TERT RNA.

Example 1: Identification of Potential Target Sites in Human TERT RNA

The sequence of human TERT was screened for accessible sites using a computer

20   folding algorithm. Regions of the RNA that did not form secondary folding structures and contained potential ribozyme and/or antisense binding/cleavage sites were identified. The sequences of these cleavage sites are shown in **tables III-VII.**

Example 2: Selection of Enzymatic Nucleic Acid Cleavage Sites in Human TERT RNA

To test whether the sites predicted by the computer-based RNA folding algorithm

25   corresponded to accessible sites in TERT RNA, 10 hammerhead ribozyme and three G-Cleaver ribozyme sites were selected for further analysis (Table VI). Ribozyme target sites were chosen by analyzing sequences of Human TERT (Nakamura et al., 1997 Science 277, 955-959; Genbank sequence accession number: NM_003219) and prioritizing the sites on the basis of folding. Ribozymes were designed that could bind

30   each target and were individually analyzed by computer folding (Christoffersen et al.,

1994 *J. Mol. Struc. Theochem,* 311, 273; Jaeger *et al.,* 1989, *Proc. Natl. Acad. Sci. USA,* **86,** 7706) to assess whether the ribozyme sequences fold into the appropriate secondary structure. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core were eliminated from consideration. As noted

5 below, varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Example 3: Chemical Synthesis and Purification of Ribozymes for Efficient Cleavage of TERT RNA

Ribozymes were designed to anneal to various sites in the RNA message. The

10 binding arms are complementary to the target site sequences described above. The ribozymes were chemically synthesized. The method of synthesis used followed the procedure for normal RNA synthesis as described above and in Usman et al., (1987 J. Am. Chem. Soc., 109, 7845), Scaringe et al., (1990 Nucleic Acids Res., 18, 5433) and Wincott et al., supra, and made use of common nucleic acid protecting and coupling

15 groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were >98%.

Ribozymes were also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, Methods Enzymol. 180, 51). Ribozymes were purified by gel electrophoresis using general methods or were purified

20 by high pressure liquid chromatography (HPLC; See Wincott et al., supra; the totality of which is hereby incorporated herein by reference) and were resuspended in water. The sequences of the chemically synthesized ribozymes used in this study are shown below in **Table III-VII.**

Example 4: Ribozyme Cleavage of TERT RNA Target *in vitro*

25 Ribozymes targeted to the human TERT RNA are designed and synthesized as described above. These ribozymes can be tested for cleavage activity *in vitro,* for example using the following procedure. The target sequences and the nucleotide location within the TERT RNA are given in Tables III-VII.

Cleavage Reactions: Full-length or partially full-length, internally-labeled target

30 RNA for ribozyme cleavage assay is prepared by *in vitro* transcription in the presence of $[a-^{32}P]$ CTP, passed over a G 50 Sephadex column by spin chromatography and used as

substrate RNA without further purification. Alternately, substrates are $5'$-^{32}P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed by pre-warming 15 µl of a 2X concentration of purified ribozyme in ribozyme cleavage buffer (50 mM Tris-HCl, pH 7.5 at 37°C, 10 mM $MgCl_2$) and the cleavage reaction was initiated by

5   adding the 2X ribozyme mix to an equal volume (15 µl) of substrate RNA (maximum of 1-5 nM; 5 x 10^5 to 1 x 10^7 cpm) that was also pre-warmed in cleavage buffer. As an initial screen, assays are carried out for 1 hour at $37°C$ using a final concentration of either 40 nM or 1 mM ribozyme, *i.e.*, ribozyme excess. The reaction is quenched by the addition of an equal volume (30 µl) of 95% formamide, 20 mM EDTA, 0.05%

10 bromophenol blue and 0.05% xylene cyanol after which the sample is heated to $95°C$ for 2 minutes, quick chilled and loaded onto a denaturing polyacrylamide gel. Substrate RNA and the specific RNA cleavage products generated by ribozyme cleavage are visualized on an autoradiograph of the gel. The percentage of cleavage is determined by Phosphor Imager[®] quantitation of bands representing the intact substrate and the

15 cleavage products.

Cell Culture Models

Various methods have been developed to assay telomerase activity *in vitro*. The most widely used method to characterize telomerase activity is the telomeric repeat amplification protocol (TRAP). TRAP utilizes RT-PCR of cellular extracts to measure

20 telomerase activity by making the amount of PCR target dependant upon the biochemical activity of the enzyme (Kim, N. W., 1997, Nucleic Acids Research, 25, 2595-2597).

Human cell culture studies have been established to assay inhibition of telomerase activity in human carcinomas responding to various therapeutics. A human breast

25 cancer model for studying telomerase inhibitors is described (Raymond, E., 1999, Br. J. Cancer, 80, 1332-1341). Human studies of telomerase expression as related to various other cancers are described including cervical cancer (Nakano, K., 1998, Am. J. Pathol, 153, 857-864), endometrial cancer (Kyo, S., 1999, Int. J. Cancer, 80, 60-63), meningeal carcinoma (Kleinschmidt-DeMasters, B. K., 1998, J. Neurol. Sci., 161, 124-134), lung

30 carcinoma (Yashima, K., 1997, Cancer Reseach, 57, 2372-2377), testicular cancer in response to cisplatin (Burger, A. M., 1997, Eur. J. Cancer, 33, 638-644), and ovarian carcinoma (Counter, C. M., 1994, Proc. Natl. Acad. Sci., 91, 2900-2904).

Animal Models

A variety of animal models have been designed to assay telomerase activity *in vivo*. Inhibition of telomerase activity has been analyzed in rats via cell proliferation studies with MNU (N-methyl-N-nitosurea) induced mammary carcinomas in response

5 to treatment with 4-(hydroxyphenyl)retinamide (4-HPR), a known inhibitor of mammary carcinogenesis in animal models and premenopausal women (Bednarek, A., 1999, Carcinogenesis, 20, 879-883). The method of Bednarek et al. uses N-methyl-N-nitrosourea (MNU)-induced mammary carcinomas in rats to analyze the effect of telomerase inhibitors *in vivo*. MNU-induced tumors express high telomerase activity.

10 Female virgin Sprague-Dawley rats are injected twice with MNU (50 mg/kg body weight) at days 43 and 50 days of age. Mammary tumors are allowed to grow to 4-8 mm before commencing treatment with an agent, such as 4-(hydroxyphenyl) retinamide (used by Bednarek *et al.*) or a nucleic acid of the invention being tested as a modulator of telomerase activity. Following treatment with an agent for 0 to 6 weeks, telomerase

15 activity is assayed using the TRAP method on CHAPS-extracted tumor-cell protein samples. A decrease of 10% or more in telomerase activity relative to the level in tumors of untreated animals indicates an agent is a telomerase inhibitor. Additional studies have focused on the up-regulation of telomerase in transformed cell lines from animal and human model systems (Zhang, P. B., 1998, Leuk. Res., 22, 509-516),

20 (Chadeneau, C., 1995, Oncogene, 11, 893-898), (Greenberg, R., 1999, Oncogene, 18, 1219-1226).

Indications

Particular degenerative and disease states that can be associated with telomerase expression modulation include but are not limited to:

25 • Cancer: Almost all human tumors have detectable telomerase activity (Shay, J. W., 1997, Eur. J. Cancer, 33, 787-791). Treatment with telomerase inhibitors may provide effective cancer therapy with minimal side effects in normal somatic cells that lack telomerase activity. The therapeutic potential exists for the treatment of a wide variety of cancer types.

30 • Restinosis: Telomerase inhibition in vascular smooth muscle cells may inhibit restinosis by limiting proliferation of these cells.

- Infectious disease: Telomerase inhibition in infectious cell types that express telomerase activity may provide selective antibiotic activity. Such treatment may prove especially effective in protozoan-based infection such as Giardia and Leishmaniasis.

- Transplant rejection: Telomerase inhibition in endothelial cell types may demonstrate selective immunnosuppressant activity. Activation of telomerase in transplant cells could benefit grafting success through increased proliferative potential.

- Autoimmune disease: Telomerase modulation in various immune cells may prove beneficial in treating diseases such as multiple sclerosis, lupus, and AIDS.

- Age related disease: Activation of telomerase expression in cells at or nearing senescence as a result of advanced age or premature aging could benefit conditions such as macular degeneration, skin ulceration, and rheumatoid arthritis.

The present body of knowledge in telomerase research indicates the need for methods to assay telomerase activity and for compounds that can regulate telomerase expression for research, diagnostic, and therapeutic use.

Gemcytabine and cyclophosphamide are non-limiting examples of chemotherapeutic agents that can be combined with or used in conjunction with the nucleic acid molecules (e.g. ribozymes and antisense molecules) of the instant invention. Those skilled in the art will recognize that other drugs such as anti-cancer compounds and therapies can be similarly be readily combined with the nucleic acid molecules of the instant invention (e.g. ribozymes and antisense molecules) and are hence within the scope of the instant invention. Such compounds and therapies are well known in the art (see for example Cancer: Principles and Practice of Oncology, Volumes 1 and 2, eds Devita, V.T., Hellman, S., and Rosenberg, S.A., J.B. Lippincott Company, Philadelphia, USA; incorporated herein by reference) and include, without limitations, antifolates; fluoropyrimidines; cytarabine; purine analogs; adenosine analogs; amsacrine; topoisomerase I inhibitors; anthrapyrazoles; retinoids; antibiotics such as bleomycin, anthacyclins, mitomycin C, dactinomycin, and mithramycin; hexamethylmelamine; dacarbazine; l-asperginase; platinum analogs; alkylating agents such as nitrogen mustard, melphalan, chlorambucil, busulfan, ifosfamide, 4-hydroperoxycyclophosphamide, nitrosoureas, thiotepa; plant derived compounds such as

vinca alkaloids, epipodophyllotoxins, taxol; Tomaxifen; radiation therapy; surgery; nutritional supplements; gene therapy; radiotherapy such as 3D-CRT; immunotoxin therapy such as ricin, monoclonal antibodies herceptin; and the like. For combination therapy, the nucleic acids of the invention are prepared in one of two ways. First, the

5　　agents are physically combined in a preparation of nucleic acid and chemotherapeutic agent, such as a mixture of a nucleic acid of the invention encapsulated in liposomes and ifosfamide in a solution for intravenous administration, wherein both agents are present in a therapeutically effective concentration (e.g., ifosfamide in solution to deliver 1000-1250 mg/m2/day and liposome-associated nucleic acid of the invention in

10　　the same solution to deliver 0.1-100 mg/kg/day).　Alternatively, the agents are administered separately but simultaneously in their respective effective doses (e.g., 1000-1250 mg/m2/d ifosfamide and 0.1 to 100 mg/kg/day nucleic acid of the invention).

Diagnostic uses

The nucleic acid molecules of this invention (*e.g., ribozymes*) may be used as

15　　diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of TERT RNA in a cell. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA.　By using multiple ribozymes described in this invention, one may map

20　　nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease.　In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of

25　　the disease progression by affording the possibility of combinational therapies (*e.g.,* multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).　Other *in vitro* uses of ribozymes of this invention are well known in the art, and include detection of the presence of mRNAs

30　　associated with TERT-related condition.　Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type

5   and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population.   Thus each analysis will require two ribozymes, two substrates and one

10 unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNAse protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. For example, the cleavage reactions are performed in ribozyme cleavage buffer with a final reaction volume of 30 µl per reaction as follows: 1) ribozyme specific for (*i.e.*, that

15 specifically cleaves) wild-type RNA (*wt* ribozyme; 40 nM final reaction concentration) is incubated with wild type substrate (1-5 nM final reaction concentration) at 37°C for one hour; 2) wt ribozyme is incubated with mutant substrate (same conditions); 3) wt ribozyme (40 nM final concentration) is incubated with 50 µg of total RNA from the individual being tested, at 37°C for one hour; 4) same as (1), only with 40 nM final

20 concentration of ribozyme specific for mutant RNA; 5) same as (2), only with ribozyme specific for mutant RNA; and 6) same as (3), only with ribozyme specific for mutant RNA.   Cleavage products are precipitated with ethanol and resuspended in 20 µl of hybridization buffer for RNAse protection with 5 x 10^5 to 1 x 10^7 cpm of ^{32}P-labeled RNA probe.   Hybridization buffer consists of the following (per reaction): 24µl

25 Formamide, 2µl 0.6M PIPES, 2.4µl 5M NaCl , 0.3µl 0.1M EDTA, and DEPC-treated water to 30 µl. Samples are heated at 95°C for 10 minutes, then incubated 4 hours at 55°C (hybridization temperatures may be estimated by one of skill in the art and optimized empirically for a given probe:target combination without undue experimentation).   Following hybridization, hybridized sequences are digested with

30 ribonucleases by the addition of 350 µl of RNase digestion buffer (300 mM NaOAc, 10 mM Tris, 5 mM EDTA) followed by addition of 1 µl of 4mg/ml RNase A and 0.4 µl of 10u/µl RNase T1. Digestion is carried out for 45 minutes to 1 hour at 30°C, followed by the addition of 10 µl of 20% SDS and 2.5 µl of 10mg/ml Proteinase K. Samples are incubated at 37°C for 15-20 minutes followed by phenol/chloroform/isoamyl alcohol

35 (25:24:1) extraction and precipitation with ethanol.   Samples are resuspended in

formamide loading buffer, heat denatured and electrophoresed on a denaturing polyacrylamide gel. Protected cleavage products are visualized by autoradiography and quantitated by phosphorimager analysis. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, TERT) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Additional Uses

Potential usefulness of sequence-specific enzymatic nucleic acid molecules of the instant invention might have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be specifically cleaved to fragments of a size more useful for study. The ability to engineer sequence specificity of the enzymatic nucleic acid molecule is ideal for cleavage of RNAs of unknown sequence. Applicant describes the use of nucleic acid molecules to down-regulate gene expression of target genes in bacterial, microbial, fungal, viral, and eukaryotic systems including plant, or mammalian cells.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to

those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Other embodiments are within the following claims.

Table 1

48

TABLE I

Characteristics of naturally occurring ribozymes

Group I Introns

- Size: ~150 to >1000 nucleotides.
- Requires a U in the target sequence immediately 5' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site.
- Reaction mechanism: attack by the 3'-OH of guanosine to generate cleavage products with 3'-OH and 5'-guanosine.
- Additional protein cofactors required in some cases to help folding and maintainance of the active structure.
- Over 300 known members of this class. Found as an intervening sequence in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.
- Major structural features largely established through phylogenetic comparisons, mutagenesis, and biochemical studies [i,ii].
- Complete kinetic framework established for one ribozyme [iii,iv,v,vi].
- Studies of ribozyme folding and substrate docking underway [vii,viii,ix].
- Chemical modification investigation of important residues well established [x,xi].
- The small (4-6 nt) binding site may make this ribozyme too non-specific for targeted RNA cleavage, however, the Tetrahymena group I intron has been used to repair a "defective" β-galactosidase message by the ligation of new β-galactosidase sequences onto the defective message [xii].

RNAse P RNA (M1 RNA)

- Size: ~290 to 400 nucleotides.
- RNA portion of a ubiquitous ribonucleoprotein enzyme.
- Cleaves tRNA precursors to form mature tRNA [xiii].
- Reaction mechanism: possible attack by M^{2+}-OH to generate cleavage products with 3'-OH and 5'-phosphate.
- RNAse P is found throughout the prokaryotes and eukaryotes. The RNA subunit has been sequenced from bacteria, yeast, rodents, and primates.
- Recruitment of endogenous RNAse P for therapeutic applications is possible through hybridization of an External Guide Sequence (EGS) to the target RNA [xiv,xv]
- Important phosphate and 2' OH contacts recently identified [xvi,xvii]

Group II Introns

- Size: >1000 nucleotides.
- Trans cleavage of target RNAs recently demonstrated [xviii,xix].
- Sequence requirements not fully determined.
- Reaction mechanism: 2'-OH of an internal adenosine generates cleavage products with 3'-OH and a "lariat" RNA containing a 3'-5' and a 2'-5' branch point.

Table 1 ● 49 ●

- Only natural ribozyme with demonstrated participation in DNA cleavage [xx,xxi] in addition to RNA cleavage and ligation.
- Major structural features largely established through phylogenetic comparisons [xxii].
- Important 2' OH contacts beginning to be identified [xxiii]
- Kinetic framework under development [xxiv]

Neurospora VS RNA

- Size: ~144 nucleotides.
- Trans cleavage of hairpin target RNAs recently demonstrated [xxv].
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined.
- Only 1 known member of this class. Found in Neurospora VS RNA.

Hammerhead Ribozyme
(see text for references)
- Size: ~13 to 40 nucleotides.
- Requires the target sequence UH immediately 5' of the cleavage site.
- Binds a variable number nucleotides on both sides of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent.
- Essential structural features largely defined, including 2 crystal structures [xxvi,xxvii]
- Minimal ligation activity demonstrated (for engineering through *in vitro* selection) [xxviii]
- Complete kinetic framework established for two or more ribozymes [xxix].
- Chemical modification investigation of important residues well established [xxx].

Hairpin Ribozyme

- Size: ~50 nucleotides.
- Requires the target sequence GUC immediately 3' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 3 known members of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent.
- Essential structural features largely defined [xxxi,xxxii,xxxiii,xxxiv]
- Ligation activity (in addition to cleavage activity) makes ribozyme amenable to engineering through *in vitro* selection [xxxv]
- Complete kinetic framework established for one ribozyme [xxxvi].
- Chemical modification investigation of important residues begun [xxxvii,xxxviii].

Table 1

50

Hepatitis Delta Virus (HDV) Ribozyme

- Size: ~60 nucleotides.
- Trans cleavage of target RNAs demonstrated [xxxix].
- Binding sites and structural requirements not fully determined, although no sequences 5' of cleavage site are required. Folded ribozyme contains a pseudoknot structure [xl].
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Only 2 known members of this class. Found in human HDV.
- Circular form of HDV is active and shows increased nuclease stability [xli]

i .   Michel, Francois; Westhof, Eric. Slippery substrates. Nat. Struct. Biol. (1994), 1(1), 5-7.

ii .   Lisacek, Frederique; Diaz, Yolande; Michel, Francois. Automatic identification of group I intron cores in genomic DNA sequences. J. Mol. Biol. (1994), 235(4), 1206-17.

iii .   Herschlag, Daniel; Cech, Thomas R.. Catalysis of RNA cleavage by the Tetrahymena thermophila ribozyme. 1. Kinetic description of the reaction of an RNA substrate complementary to the active site. Biochemistry (1990), 29(44), 10159-71.

iv .   Herschlag, Daniel; Cech, Thomas R.. Catalysis of RNA cleavage by the Tetrahymena thermophila ribozyme. 2. Kinetic description of the reaction of an RNA substrate that forms a mismatch at the active site. Biochemistry (1990), 29(44), 10172-80.

v .   Knitt, Deborah S.; Herschlag, Daniel. pH Dependencies of the Tetrahymena Ribozyme Reveal an Unconventional Origin of an Apparent pKa. Biochemistry (1996), 35(5), 1560-70.

vi .   Bevilacqua, Philip C.; Sugimoto, Naoki; Turner, Douglas H.. A mechanistic framework for the second step of splicing catalyzed by the Tetrahymena ribozyme. Biochemistry (1996), 35(2), 648-58.

vii .   Li, Yi; Bevilacqua, Philip C.; Mathews, David; Turner, Douglas H.. Thermodynamic and activation parameters for binding of a pyrene-labeled substrate by the Tetrahymena ribozyme: docking is not diffusion-controlled and is driven by a favorable entropy change. Biochemistry (1995), 34(44), 14394-9.

viii .   Banerjee, Aloke Raj; Turner, Douglas H.. The time dependence of chemical modification reveals slow steps in the folding of a group I ribozyme. Biochemistry (1995), 34(19), 6504-12.

ix .   Zarrinkar, Patrick P.; Williamson, James R.. The P9.1-P9.2 peripheral extension helps guide folding of the Tetrahymena ribozyme. Nucleic Acids Res. (1996), 24(5), 854-8.

x .   Strobel, Scott A.; Cech, Thomas R.. Minor groove recognition of the conserved G.cntdot.U pair at the Tetrahymena ribozyme reaction site. Science (Washington, D. C.) (1995), 267(5198), 675-9.

xi .   Strobel, Scott A.; Cech, Thomas R.. Exocyclic Amine of the Conserved G.cntdot.U Pair at the Cleavage Site of the Tetrahymena Ribozyme Contributes to 5'-Splice Site Selection and Transition State Stabilization. Biochemistry (1996), 35(4), 1201-11.

xii.   Sullenger, Bruce A.; Cech, Thomas R.. Ribozyme-mediated repair of defective mRNA by targeted trans-splicing. Nature (London) (1994), 371(6498), 619-22.

xiii.   Robertson, H.D.; Altman, S.; Smith, J.D. J. Biol. Chem., 247, 5243-5251 (1972).

xiv.   Forster, Anthony C.; Altman, Sidney. External guide sequences for an RNA enzyme. Science (Washington, D. C., 1883-) (1990), 249(4970), 783-6.

xv.   Yuan, Y.; Hwang, E. S.; Altman, S. Targeted cleavage of mRNA by human RNase P. Proc. Natl. Acad. Sci. USA (1992) 89, 8006-10.

xvi .   Harris, Michael E.; Pace, Norman R.. Identification of phosphates involved in catalysis by the ribozyme RNase P RNA. RNA (1995), 1(2), 210-18.

xvii .   Pan, Tao; Loria, Andrew; Zhong, Kun. Probing of tertiary interactions in RNA: 2'-hydroxyl-base contacts between the RNase P RNA and pre-tRNA. Proc. Natl. Acad. Sci. U. S. A. (1995), 92(26), 12510-14.

xviii .   Pyle, Anna Marie; Green, Justin B.. Building a Kinetic Framework for Group II Intron Ribozyme Activity: Quantitation of Interdomain Binding and Reaction Rate. Biochemistry (1994), 33(9), 2716-25.

xix .   Michels, William J. Jr.; Pyle, Anna Marie. Conversion of a Group II Intron into a New Multiple-Turnover Ribozyme that Selectively Cleaves Oligonucleotides: Elucidation of Reaction Mechanism and Structure/Function Relationships. Biochemistry (1995), 34(9), 2965-77.

xx .   Zimmerly, Steven; Guo, Huatao; Eskes, Robert; Yang, Jian; Perlman, Philip S.; Lambowitz, Alan M.. A group II intron

Table 1

51

RNA is a catalytic component of a DNA endonuclease involved in intron mobility. Cell (Cambridge, Mass.) (1995), 83(4), 529-38.

[xxi].   Griffin, Edmund A., Jr.; Qin, Zhifeng; Michels, Williams J., Jr.; Pyle, Anna Marie. Group II intron ribozymes that cleave DNA and RNA linkages with similar efficiency, and lack contacts with substrate 2'-hydroxyl groups. Chem. Biol. (1995), 2(11), 761-70.

[xxii].   Michel, Francois; Ferat, Jean Luc Structure and activities of group II introns. Annu. Rev. Biochem. (1995), 64, 435-61.

[xxiii].   Abramovitz, Dana L.; Friedman, Richard A.; Pyle, Anna Marie. Catalytic role of 2'-hydroxyl groups within a group II intron active site. Science (Washington, D. C.) (1996). 271(5254), 1410-13.

[xxiv].   Daniels, Danette L.; Michels, William J., Jr.; Pyle, Anna Marie. Two competing pathways for self-splicing by group II introns: a quantitative analysis of in vitro reaction rates and products. J. Mol. Biol. (1996), 256(1), 31-49.

[xxv].   Guo, Hans C. T.; Collins, Richard A.. Efficient trans-cleavage of a stem-loop RNA substrate by a ribozyme derived from Neurospora VS RNA. EMBO J. (1995), 14(2), 368-76.

[xxvi].   Scott, W.G., Finch, J.T., Aaron,K. The crystal structure of an all RNA hammerhead ribozyme:Aproposed mechanism for RNA catalytic cleavage. Cell, (1995), 81, 991-1002.

[xxvii].   McKay, Structure and function of the hammerhead ribozyme: an unfinished story. RNA, (1996), 2, 395-403.

[xxviii].   Long, D., Uhlenbeck, O., Hertel, K. Ligation with hammerhead ribozymes. US Patent No. 5,633,133.

[xxix].   Hertel, K.J., Herschlag, D., Uhlenbeck, O. A kinetic and thermodynamic framework for the hammerhead ribozyme reaction. Biochemistry, (1994) 33, 3374-3385.Beigelman, L., *et al.*, Chemical modifications of hammerhead ribozymes. J. Biol. Chem., (1995) 270, 25702-25708.

[xxx].   Beigelman, L., *et al.*, Chemical modifications of hammerhead ribozymes. J. Biol. Chem., (1995) 270, 25702-25708.

[xxxi].   Hampel, Arnold; Tritz, Richard; Hicks, Margaret; Cruz, Phillip. 'Hairpin' catalytic RNA model: evidence for helixes and sequence requirement for substrate RNA. Nucleic Acids Res. (1990), 18(2), 299-304.

[xxxii].   Chowrira, Bharat M.; Berzal-Herranz, Alfredo; Burke, John M.. Novel guanosine requirement for catalysis by the hairpin ribozyme. Nature (London) (1991), 354(6351), 320-2.

[xxxiii].   Berzal-Herranz, Alfredo; Joseph, Simpson; Chowrira, Bharat M.; Butcher, Samuel E.; Burke, John M.. Essential nucleotide sequences and secondary structure elements of the hairpin ribozyme. EMBO J. (1993), 12(6), 2567-73.

[xxxiv].   Joseph, Simpson; Berzal-Herranz, Alfredo; Chowrira, Bharat M.; Butcher, Samuel E.. Substrate selection rules for the hairpin ribozyme determined by in vitro selection, mutation, and analysis of mismatched substrates. Genes Dev. (1993), 7(1), 130-8.

[xxxv].   Berzal-Herranz, Alfredo; Joseph, Simpson; Burke, John M.. In vitro selection of active hairpin ribozymes by sequential RNA-catalyzed cleavage and ligation reactions. Genes Dev. (1992), 6(1), 129-34.

[xxxvi].   Hegg, Lisa A.; Fedor, Martha J.. Kinetics and Thermodynamics of Intermolecular Catalysis by Hairpin Ribozymes. Biochemistry (1995), 34(48), 15813-28.

[xxxvii].   Grasby, Jane A.; Mersmann, Karin; Singh, Mohinder; Gait, Michael J.. Purine Functional Groups in Essential Residues of the Hairpin Ribozyme Required for Catalytic Cleavage of RNA. Biochemistry (1995), 34(12), 4068-76.

[xxxviii].   Schmidt, Sabine; Beigelman, Leonid; Karpeisky, Alexander; Usman, Nassim; Sorensen, Ulrik S.; Gait, Michael J.. Base and sugar requirements for RNA cleavage of essential nucleoside residues in internal loop B of the hairpin ribozyme: implications for secondary structure. Nucleic Acids Res. (1996), 24(4), 573-81.

[xxxix].   Perrotta, Anne T.; Been, Michael D.. Cleavage of oligoribonucleotides by a ribozyme derived from the hepatitis .delta. virus RNA sequence. Biochemistry (1992), 31(1), 16-21.

[xl].   Perrotta, Anne T.; Been, Michael D.. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature (London) (1991), 350(6317), 434-6.

[xli].   Puttaraju, M.; Perrotta, Anne T.; Been, Michael D.. A circular trans-acting hepatitis delta virus ribozyme. Nucleic Acids Res. (1993), 21(18), 4253-8.

Table II 52

Table II: 0.2 µmol RNA Synthesis Cycle

Reagents	Equivalents	Amounts (microL)	Wait time (sec)
Phosphoramidites	15	31	465
SET	38.7	31	465
Acetic anhydride	655	124	5
N-methyl-imidazole	1245	124	5
TCA	700	732	10
Iodine	20.6	244	15

* Wait time does not include contact time during delivery.